Evaluation of the Influence of Bacteriophage Titer on the Treatment of Colibacillosis in Broiler Chickens[1]

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ABSTRACT Two studies were conducted to determine the efficacy of bacteriophage SPR02 and DAF6 at varying titers to treat colibacillosis in chickens. In Study 1, the treatments consisted of a control, i.m. injection of bacteriophage SPR02 or DAF6, *Escherichia coli* airsac challenge, and *E. coli* challenge followed by treatment at different titers with bacteriophage SPR02 or DAF6. The *E. coli*-challenged birds were injected with 6×10^4 cfu into the left thoracic airsac at 7 d of age. Immediately after the birds were challenged with *E. coli*, they were treated by administration of bacteriophage SPR02 or DAF6 by i.m. injection into the left thigh with 4×10^8, 10^6, 10^4, or 10^2 pfu. Study 2 was identical to Study 1, with the exception that the E. coli challenge was increased to 9×10^4 cfu, and the titers of SPR02 and DAF6 were slightly less at 3 $\times 10^8$, 10^6, 10^4, and 10^2 pfu. Both studies were concluded when the birds were 3 wk of age. Mortality in the birds challenged with *E. coli* in Studies 1 and 2 was 48 and 47%, respectively. The only consistently effective bacteriophage treatment was the highest titer (10^8 pfu) of bacteriophage SPR02, which significantly reduced mortality from 48 and 47% in the birds only challenged with *E. coli* (positive control) to 7% in both studies, which was not significantly different from the unchallenged negative control treatments. These studies indicate that an effective multiplicity of infection for i.m. treatment with SPR02 was 10^4 in this experimental model of colibacillosis. Bacteriophage administered at sufficient titers can be effective therapeutic agents and provide an alternative to antibiotics in the treatment of bacterial diseases.

Key words: bacteriophage therapy, *Escherichia coli*, chicken, colibacillosis

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INTRODUCTION

Bacteriophage are viruses that infect, replicate within, and kill bacteria by lysis. Upon lysis of the bacteria, a large number of active bacteriophage are released that are capable of infecting and killing additional bacteria. This life cycle of lytic bacteriophage being self-replicating and self-limiting makes them attractive as a natural and safe alternative to antibiotics to both prevent and treat bacterial diseases. Indeed, our research exploring the use of bacteriophage to prevent and treat an *Escherichia coli* respiratory infection suggests that bacteriophage may provide an alternative to antibiotics in poultry production for the prevention and therapeutic treatment of poultry diseases (Huff et al., 2002a,b; 2003a,b). Our previous research on using bacteriophage to treat colibacillosis used a combination of 2 bacteriophage at relatively high bacteriophage titers, approximately 10^8 pfu. The objective of this research was to evaluate the efficacy of various titers of these 2 bacteriophage administered individually.

MATERIALS AND METHODS

Bacteriophage Isolation and Amplification

Bacteriophage were isolated to an *E. coli* poultry isolate that is nonmotile, lactose negative, serotype 02 using either waste water from municipal sewer treatment plants or a poultry processing plant as described by Huff et al. (2002b). Two bacteriophage isolates, designated SPR02 and DAF6, were selected for these studies based on size and clarity of plaques. The bacteriophage were amplified and numerated by procedures previously detailed (Huff et al., 2002b).

E. coli Challenge Culture

The *E. coli* used in these studies were initially isolated from the blood of chickens with colisepticemia (Bayyari et al., 1997; Huff et al., 1998). This *E. coli* strain is serotype 02, nonmotile, and lactose negative. The *E. coli* culture was prepared by inoculation of tryptose phosphate broth (Sigma Chemical Co., St. Louis, MO) that was incubated

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in a shaking water bath for 2.5 h. The culture was removed from the water bath and held at 4°C. The culture was enumerated by making duplicate 10-fold serial dilutions of the culture and by spread-plating the appropriate dilutions in duplicate on tryptose phosphate agar plates, which were enumerated after overnight incubation at 37°C. The challenge cultures were made by diluting this *E. coli* stock culture, and verified with serial dilutions of the challenge culture and enumeration by spread plating.

Experimental Design

Two studies were conducted to determine the efficacy of 2 separate bacteriophage at varying titers to treat a severe *E. coli* respiratory infection. For both studies, male broiler chicks (Cobb 500) were obtained from a local hatchery and maintained in electrically heated batteries with feed and chlorinated water available for ad libitum consumption to 3 wk of age when the studies were concluded. The birds that were challenged with *E. coli* were maintained in separate rooms. Study 1 consisted of 12 treatments with 3 replicate pens of 10 birds per pen. The treatments consisted of a negative control (unchallenged), birds administered only the bacteriophage SPR02 or DAF6, birds challenged only with *E. coli* (positive control), and birds challenged with *E. coli* and administered decreasing titers of either bacteriophage SPR02 or DAF6. The *E. coli*-challenged birds were injected with 0.1 mL of a 2.5-h culture of *E. coli* containing 6×10^5 cfu/mL providing 6×10^4 cfu into the left thoracic airsac at 7 d of age. Immediately after the birds were challenged with *E. coli*, they were treated by administration of 0.1 mL of bacteriophage SPR02 or DAF6 preparation containing 4×10^9, 10^7, 10^5, or 10^3 pfu/mL injected i.m. into the left thigh, providing 4×10^8, 10^6, 10^4, or 10^2 pfu. Study 2 was identical to Study 1, with the exception that the *E. coli* challenge dose was increased to 9×10^4 cfu, and the titers of SPR02 and DAF6 were slightly less at 3×10^8, 10^6, 10^4, and 10^2 pfu.

The birds were individually weighed each week. Any bird that died was weighed, the severity of airsaculitis was scored (Huff et al., 1998), and the liver and airsac were cultured with sterile transport swabs, and plated on MacConkey's agar (Remel, Lenexa, KS). The liver, heart, spleen, and bursa of Fabricius were excised and weighed. When the birds were 3 wk of age, they were humanely euthanized by cervical dislocation and necropsied as described above. All procedures described in these studies were approved by the University of Arkansas Animal Care and Use Committee.

Statistical Analysis

These data were analyzed by ANOVA (Snedecor and Cochran, 1967) using the GLM procedures of SAS software (SAS Institute Inc., 1998). All data presented as percentages were transformed as the square root of the arc sine prior to statistical analysis. Pen means were the unit for statistical analysis. For ease and clarity of presentation, the statistical analysis of the effects of each bacteriophage were analyzed separately using the common control. Significant differences among treatments were separated using Duncan's multiple range test (Duncan, 1955). All statements of significance are based on the probability level of 0.05.

Table 1. The effect of SPR02 bacteriophage i.m treatment at varying titers on mortality of birds unchallenged and challenged with an airsac inoculation of *Escherichia coli* [6×10^4 cfu (Study 1) and 9×10^4 cfu (Study 2)][1]

	Mortality (%)	
Treatment	Study 1	Study 2
Control	0 ± 0[c]	0 ± 0[c]
SPR02 10^8 pfu	0 ± 0[c]	3 ± 3[bc]
E. coli challenge	48 ± 7[a]	47 ± 15[a]
SPR02 10^8 pfu *E. coli* challenge	7 ± 7[c]	7 ± 3[bc]
SPR02 10^6 pfu *E. coli* challenge	24 ± 3[ab]	13 ± 3[b]
SPR02 10^4 pfu *E. coli* challenge	10 ± 6[bc]	50 ± 6[a]
SPR02 10^2 pfu *E. coli* challenge	28 ± 4[a]	53 ± 9[a]

[a–c]Values within a column with different superscripts differ significantly ($P \leq 0.05$).

[1]Values represent the mean ± SEM of 3 replicate pens of 10 birds per pen.

RESULTS

The effects of varying titers of bacteriophage SPR02 on mortality in Studies 1 and 2 are presented in Table 1. In Study 1, there was 48% mortality in the birds challenged with *E. coli* and not treated with bacteriophage. Mortality in the birds treated with the SPR02 10^8 and 10^4 pfu treatments were 7 and 10%, respectively, and were significantly different from the *E. coli* treatment and not significantly different from the control. Mortality in birds in the SPR02 treatments of 10^6 and 10^2 pfu were 24 and 28%, respectively, which were not significantly different from the birds challenged with *E. coli* and not treated with bacteriophage (48%). In Study 2, mortality in the birds challenged with *E. coli* and not treated with bacteriophage was 47%. The mortality observed in the SPR02 treatments of 10^8 and 10^6 pfu were 7 and 13%, respectively, which were significantly different from the birds challenged with *E. coli* and not treated with bacteriophage (47%) and not significantly different from the control treatment. The mortality in the SPR02 10^4 and 10^2 pfu treatments was not significantly different from the mortality in the birds challenged with *E. coli* and not treated with bacteriophage.

The effects on mortality using varying titers of bacteriophage DAF6 in Studies 1 and 2 are presented in Table 2. The only bacteriophage treatment in both studies that showed any therapeutic efficacy to treat colibacillosis was DAF6 at 10^6 pfu in Study 1; the mortality in birds receiving all other DAF6 treatments in both studies was not significantly different from the birds challenged with *E. coli* and untreated.

Body weights of the birds given bacteriophage only were not significantly different from the control (data not shown). Necropsy results of birds that died were consistent with colibacillosis lesions characterized by air-

Table 2. The effect of DAF6 bacteriophage i.m. treatment at varying titers on mortality of birds unchallenged and challenged with an airsac inoculation of *Escherichia coli* [6×10^4 cfu (Study 1) and 9×10^4 cfu (Study 2)][1]

Treatment	Mortality (%)	
	Study 1	Study 2
Control	0 ± 0^{c}	0 ± 0^{b}
DAF6 10^8 pfu	0 ± 0^{c}	3 ± 3^{b}
E. coli challenge	48 ± 7^{a}	47 ± 15^{a}
DAF6 10^8 pfu *E. coli* challenge	$20 \pm 12^{a–c}$	40 ± 6^{a}
DAF6 10^6 pfu *E. coli* challenge	7 ± 3^{bc}	47 ± 3^{a}
DAF6 10^4 pfu *E. coli* challenge	$23 \pm 15^{a–c}$	47 ± 7^{a}
DAF6 10^2 pfu *E. coli* challenge	$23 \pm 7^{a–c}$	37 ± 3^{a}

[a–c]Values within a column with different superscripts differ significantly ($P \leq 0.05$).

[1]Values represent the mean ± SEM of 3 replicate pens of 10 birds per pen.

sacculitis and pericarditis; an increase in the relative weights of the liver, spleen, and heart; and a decrease in the relative weight of the bursa of Fabricius (data not shown). Our challenge strain of *E. coli* was isolated from swabs of the air sac and liver in affected birds, with over 90% of the cultures being pure; swabs taken from our control birds were culture negative (data not shown). The challenge culture of *E. coli* is a lactose-negative, nonmotile, serotype 02 that is easily identified on MacConkey agar on the basis of fermentation of lactose and colony morphology.

DISCUSSION

Bacteriophage infect bacteria, replicate within the bacterium, and from 10 to 200 active bacteriophage emerge from the killed bacteria cell. The emerging bacteriophage can, in turn, infect additional bacteria, resulting in an exponential increase in bacteriophage. These self-replicating and self-limiting aspects of bacteriophage are 2 of the many attributes of bacteriophage that make them attractive alternatives to antibiotics to prevent and treat bacterial diseases. Theoretically, bacteriophage could be administered at small doses and have therapeutic efficacy to treat bacterial diseases, given the exponential growth of bacteriophage. However, data from these studies suggest that bacteriophage SPR02 was only completely effective at treating this experimentally induced colibacillosis at titers of 10^8 pfu, consistent with the previously reported treatment efficacy (Huff et al., 2003a,b). Multiplicity of infection (**MOI**) is a ratio of the number of bacteriophage per bacteria. In the current studies, complete treatment efficacy was only achieved with SPR02 with an MOI of 10^4 (10^8 pfu of bacteriophage vs. 10^4 cfu of *E. coli*). A wide range of MOI for complete protection of animals in disease models have been reported. Soothill (1992) found that total protection of mice was achieved when both *Acinetobacter baumanii* and bacteriophage were injected i.p. at an extremely small MOI of less than 1 (10^{-6}). Cerveny et al. (2002) reported that total protection of mice was observed when *Vibrio vulnificus* was injected s.c. followed by an i.v. injection of bacteriophage at an MOI of 10^5. When *E. coli* was injected i.m. in mice, followed by either an i.m. or i.v. injection of bacteriophage, total protection of mice, based on mortality, was achieved at MOI of less than 1, 10^{-3}, and 10^{-4}, respectively. Based on mortality, Barrow et al. (1998) found that an MOI of 10^{-2} gave complete protection of chickens when *E. coli* was injected i.m. into a leg followed by an i.m. injection of bacteriophage into the other leg. However, when the *E. coli* was injected intracranially, followed by an i.m. injection of bacteriophage, total protection was achieved only with an MOI of 10^5 (Barrow et al., 1998). In studies using wild-type bacteriophage and bacteriophage selected for systemic retention, Merril et al. (1996) found that an MOI of 10^2 was effective in mice when both *E. coli* and the bacteriophage were injected i.p., with less severe symptoms observed with the bacteriophage selected for increased systemic retention. Biswas et al. (2002) reported that an MOI of 0.3 and 3 was effective in treating mice with bacteriophage challenged with *Enterococcus faecium* when both the bacteria and bacteriophage were injected i.p. Matsuzaki et al. (2003) concluded that an MOI of 1 was the minimal requirement to fully protect mice from a *Staphylococcus aureus* challenge with bacteriophage when both were administered i.p.

The MOI for complete protection of animals from bacterial diseases with bacteriophage vary from as low as 10^{-6} (Soothill, 1992) to as high as 10^5 (Cerveny et al., 2002). In general, MOI are lower in experimental models in which bacteria and bacteriophage are administered at the same site and increase as the site of administration of bacteria and bacteriophage differ anatomically. We have shown this in our own research, in which an MOI of 1 protected birds when they were airsac challenged with *E. coli* mixed with bacteriophage (Huff et al., 2002b), compared with an MOI for complete protection (in this study) of 10^4, in which the birds were challenged with an airsac inoculation of *E. coli* and treated with an i.m. administration of bacteriophage. Therefore, basing bacteriophage efficacy on MOI among experimental models of animal diseases would appear to have little value and should not be used to base bacteriophage treatment of natural diseases. However, comparing the efficacy among individual bacteriophage based on MOI within an animal disease model may have value. Bacteriophage depend on the chance meeting with bacteria to infect and kill bacteria. If bacteriophage reach the site of a bacterial infection, they are effective in eliminating the infection. When a bacterial infection is systemic, with bacteria dispersed throughout the animal, it would appear that bacteriophage treatment efficacy would be enhanced by ensuring that sufficiently large numbers of bacteriophage are present, increasing the probability of the chance meeting of bacteriophage with the targeted bacteria. Based on these considerations, bacteriophage titers should be maximized for the therapeutic treatment of natural diseases.

It was very surprising that bacteriophage DAF6 had little treatment efficacy compared with bacteriophage SPR02 in these 2 studies. Bacteriophage DAF6 in vitro completely killed the *E. coli* strain used in this study in

broth cultures and produced small, but clear, plaques in soft agar overlay plates. We have no explanation for the low treatment efficacy with DAF6 in these 2 studies. However, these data clearly demonstrate that not all bacteriophage that show in vitro efficacy will be effective as therapeutic agents in vivo.

We have been able to demonstrate that bacteriophage have the potential to be developed as effective agents to prevent and treat animal bacterial diseases (Huff et al., 2002a,b; 2003a,b). There is a continuing interest in the use of bacteriophage to control bacterial infections. The ability of bacteriophage to control *E. coli*-induced diarrhea in calves, piglets, and lambs was demonstrated by research conducted by Smith and colleagues (Smith and Huggins, 1983; Smith et al., 1987). These authors also demonstrated the ability of phage to treat *E. coli* infections in mice (Smith and Huggins, 1982). Barrow et al. (1998) demonstrated the ability of bacteriophage to protect chickens from an i.m. challenge with *E. coli*, and Soothill (1992) found that bacteriophage would protect mice from infection with *A. baumanii* and *Pseudomonas aeruginosa*. Biswas et al. (2002) demonstrated that bacteriophage could rescue mice from a lethal challenge with vancomycin-resistant *E. faecium*. Bacteriophage provided effective disease control in the ayu, a fish raised in Japan, caused by *Pseudomonas plecoglossicida* (Park et al., 2000). Matsuzaki et al. (2003) were able to protect mice from a lethal injection of *S. aureus* using bacteriophage, and Cerveny et al. (2002) were able to demonstrate that bacteriophage had therapeutic value in the treatment of both localized and systemic infections with *V. vulnificus* in a mice model. The potential use of bacteriophage in agriculture also extends to food safety applications targeting foodborne pathogens. Research on the efficacy of bacteriophage to reduce *Listeria monocytogenes* on fresh-cut produce was demonstrated by Leverentz et al. (2003). Lytic bacteriophage have been shown to decrease *Salmonella* and *Campylobacter* contamination on chicken skin (Goode et al., 2003). There is also a significant research effort being conducted on controlling *Salmonella* on poultry products (Higgins et al., 2005). It remains to be seen whether bacteriophage will be developed into cost-effective and practical products for agricultural applications due to some of the important concerns with bacteriophage that are detailed by Huff et al. (2004). However, these data suggest that there is sufficient reason to believe that bacteriophage can play a significant role in the prevention and treatment of bacterial diseases and the reduction of foodborne pathogens on agricultural products.

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